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Sunlight Inactivation of Human Viruses and Bacteriophages in Coastal Waters Containing Natural  
Photosensitizers

Andrea I. Silverman<sup>1</sup>, Britt M. Peterson<sup>2</sup>, Alexandria B. Boehm<sup>3</sup>, Kristopher McNeill<sup>2</sup>, and Kara L.  
Nelson<sup>1,\*</sup>

<sup>1</sup> Department of Civil and Environmental Engineering, University of California, Berkeley, Berkeley,  
California, United States

<sup>2</sup> Institute of Biogeochemistry and Pollutant Dynamics (IBP), ETH Zurich, Zurich, Switzerland

<sup>3</sup> Environmental and Water Studies, Department of Civil and Environmental Engineering, Stanford  
University, Stanford, California, United States

\* Corresponding Author: nelson@ce.berkeley.edu

16   **Abstract**

17   Sunlight inactivation of poliovirus type 3 (PV3), adenovirus type 2 (HAdV2), and two bacteriophage  
18   (MS2 and PRD1) was investigated in an array of coastal waters to better understand solar inactivation  
19   mechanisms and the effect of natural water constituents on observed inactivation rates ( $k_{\text{obs}}$ ). Reactor  
20   scale inactivation experiments were conducted using a solar simulator, and  $k_{\text{obs}}$  for each virus was  
21   measured in a sensitizer-free control and five unfiltered surface water samples collected from different  
22   sources.  $k_{\text{obs}}$  values varied between viruses in the same water matrix, and for each virus in different  
23   matrices, with PV3 having the fastest and MS2 the slowest  $k_{\text{obs}}$  in all waters. When exposed to full-  
24   spectrum sunlight, the presence of photosensitizers significantly increased  $k_{\text{obs}}$  of HAdV2 and MS2, but  
25   not PV3 and PRD1, which provides evidence that the exogenous sunlight inactivation mechanism,  
26   involving damage by exogenously produced reactive intermediates, played a greater role in inactivation  
27   of HAdV2 and MS2. While PV3 and PRD1 inactivation was observed to be dominated by endogenous  
28   mechanisms, this may be due to a masking of exogenous  $k_{\text{obs}}$  by significantly faster endogenous  $k_{\text{obs}}$ .  
29   Results illustrate that differences in water composition can shift absolute and relative inactivation rates  
30   of viruses, which has important implications for natural wastewater treatment systems, solar  
31   disinfection (SODIS), and the use of indicator organisms for monitoring water quality.

## Introduction

Surface water contamination by human enteric viruses is common and can occur through urban runoff<sup>1,2</sup> and wastewater flows into the environment.<sup>3,4</sup> Viruses are more difficult to remove and inactivate than fecal indicator bacteria during wastewater treatment, and infectious human viruses have been found in wastewater treatment facility effluents discharged into surface waters.<sup>5</sup> Sunlight – which contains UVB ( $\lambda=280\text{--}320\text{ nm}$ ), UVA ( $\lambda=320\text{--}400\text{ nm}$ ) and visible light ( $\lambda=400\text{--}700\text{ nm}$ ) regions – causes inactivating damage to microorganisms<sup>6-12</sup> and is an important mode of disinfection in waste stabilization ponds (WSP),<sup>6,7,13</sup> recreational water bodies,<sup>3</sup> and SODIS.<sup>14-16</sup> While viruses are a significant cause of waterborne disease, and believed to be the main etiology of disease to swimmers at recreational beaches,<sup>17</sup> sunlight inactivation of human viruses in the environment is understudied.

Most waterborne viruses are non-enveloped and consist of nucleic acids (single- or double-stranded RNA or DNA) surrounded by a protective protein capsid. The sunlight inactivation terminology presented below is a slight shift from that used previously,<sup>7,9-12,18</sup> but is introduced to better relate mechanisms to environmental conditions; a summary is presented in Table S1. There are three proposed virus inactivation mechanisms: the direct and indirect endogenous mechanisms require absorption of photons by virus components, while the exogenous mechanism involves reaction between the virus and exogenously produced reactive intermediates formed in photochemical reactions. The direct and indirect endogenous mechanisms differ in that the direct mechanism causes damage to the virus component that absorbed the photon, either nucleic acids (leading to formation of pyrimidine dimers and other photoproducts)<sup>18</sup> or proteins (resulting in photo-oxidation of select amino acid residues),<sup>19,20</sup> while the indirect-endogenous mechanism occurs when photons are absorbed by one part of the virus, but damage is conveyed to another through electron transfer or sensitized formation of and subsequent reaction with reactive intermediates, such as singlet oxygen ( $^1\text{O}_2$ ).<sup>19</sup> It is difficult to separate the two endogenous mechanisms experimentally, and they are often lumped together. The exogenous mechanism occurs when photons are absorbed by photosensitizers in the water column, leading to the formation of reactive intermediates, such as excited triplet states (e.g., triplet dissolved organic matter ( $^3\text{DOM}$ )) and reactive oxygen species (ROS; e.g.,  $^1\text{O}_2$ , hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide ( $\text{O}_2^-$ ), hydroxyl radical ( $\text{OH}\cdot$ ), and peroxy radicals).<sup>21,22</sup> Reactive intermediates contribute to inactivation by causing oxidative damage to virion components;<sup>6,7,23</sup> to date, there is only evidence of  $^1\text{O}_2$  involvement in photoinactivation of viruses.<sup>9</sup> While both the indirect-endogenous and exogenous mechanisms involve reaction with reactive intermediates, the indirect-endogenous mechanism depends on photons absorbed by endogenous sensitizers (e.g., amino acid residues),<sup>19,24</sup>

and the exogenous mechanism depends on photons absorbed by photosensitizers in the water (e.g., natural organic matter (NOM)).<sup>6,25</sup>

Many studies investigating sunlight inactivation of viruses have focused on MS2 and other F+RNA coliphage<sup>7-12,25,26</sup> – single-stranded RNA bacteriophages frequently used as model organisms for enteric viruses due to their similar structure and size<sup>27</sup> – and somatic coliphage.<sup>11,26</sup> MS2, for example, has been found to be susceptible to endogenous<sup>11</sup> and exogenous<sup>9,10</sup> inactivation mechanisms when exposed to sunlight in the presence of sensitizers. Bacteriophage, however, do not pose human health threats and may not accurately model human virus inactivation under all environmental conditions. Variable virus structures, and therefore targets of sunlight damage, may result in different rates and dominant mechanisms of sunlight-mediated inactivation. At this point, it is not possible to predict mechanisms or rates of sunlight inactivation of viruses of public health concern based on current knowledge of bacteriophage inactivation. To better understand sunlight inactivation of mammalian viruses, it is necessary to study them directly. To date, few published studies have investigated the effect of sunlight on mammalian viruses, and no previous studies have investigated mammalian virus inactivation in environmentally sourced waters containing natural sensitizers, such as NOM.

NOM is found in most aquatic environments<sup>22</sup> and, due to its high concentration of aromatic functional groups,<sup>28</sup> is one of the most important sunlight absorbing substances in natural waters.<sup>29</sup> NOM varies in composition and structure depending on its source,<sup>30</sup> which can cause NOM from different environmental waters to possess varying propensities to absorb light, produce ROS and associate with other water constituents, including viruses. Because NOM is capable of both sunlight attenuation and ROS production, NOM can increase or decrease sunlight inactivation rates, depending on its structure and the dominant mechanism of inactivation of the target organism. To investigate the variability of virus inactivation rates in different surface waters, it is important to study sunlight inactivation of viruses in waters containing natural dissolved and particulate constituents.

In this study we investigated sunlight inactivation of viruses in five environmentally sourced coastal waters containing NOM. The goals of this study were to: (1) determine the main mechanisms of sunlight inactivation of two human viruses (HAdV2 and PV3) and two bacteriophages (MS2 and PRD1) in environmental waters containing natural photosensitizers (see Table S2 for virus characteristics); (2) compare sunlight inactivation rates *between viruses*; and (3) investigate the variability in inactivation rates of each organism *between environmental matrices*. A better

understanding of sunlight-mediated disinfection mechanisms and relative rates of virus inactivation in NOM-containing waters can aid in designing natural wastewater treatment schemes, predicting rates of virus inactivation in sunlit environments, and determining the best indicator organisms for tracking the fate of viruses in surface waters, such as recreational beaches and engineered treatment systems.

## Methods

Target viruses were inoculated into reactors containing one of five unfiltered, environmentally sourced waters (with presumably different concentrations and sources of NOM) or a sensitizer-free control water; reactors were exposed to simulated sunlight and virus concentrations were monitored to determine rates of sunlight inactivation in each solution.

**Human Viruses.** Detailed virus and bacteriophage methods are provided in Love et al.<sup>11</sup> Briefly, PV3 (ATCC VR-300) was cultured on HeLa cells (ATCC CCL-2). HAdV2 was kindly provided by Mark Sobsey (University of North Carolina) and cultured on A549 cells (ATCC CCL-185). Viruses were propagated on 90% confluent cells in T-150 flasks; flasks were incubated at 37 °C and 5% CO<sub>2</sub> for 4 d (PV3) or 7 d (HAdV2) with 1X Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), 1X penicillin and streptomycin (pen/strep), and 2% (HAdV2) or 10% (PV3) fetal bovine serum (FBS). After incubation, viruses were released from cells by three freeze/thaw cycles. To remove broth constituents, crude virus stocks were chloroform extracted (1:3 vol/vol), centrifuged at 4000 ×g for 10 min to remove cell debris, and the supernatant was polyethylene glycol (PEG) precipitated overnight at 4 °C (9% PEG, 0.3 M NaCl), centrifuged at 20,000 ×g for 15 min to produce virus pellets that were resuspended in phosphate buffered saline (PBS; 10 mM NaCl, 20 mM phosphate), chloroform extracted as above and filtered through a 0.22 µm filter. Virus stocks were stored at -80 °C.

Virus plaque assays were performed in duplicate on 6-well plates of 90% confluent cells with 100 µl sample inocula and an agar overlay (1.5% wt/vol low melting point agarose (Fisher Scientific), 1X DMEM, 1X pen/strep, and 2% (HAdV2) or 10% (PV3) FBS). Plates were incubated at 37 °C and 5% CO<sub>2</sub> for 3 d (PV3) or 7 d (HAdV2) and plaque forming units (PFU) were counted. Samples were not filtered before analysis.

**Bacteriophage.** MS2 and PRD1 were kindly provided by Mark Sobsey, and were propagated and assayed using *E. coli* F<sub>amp</sub> (ATCC 700891) and *Salmonella* LT2 (ATCC 19585) hosts, respectively. Bacteriophage were propagated by broth enrichment. Purified bacteriophage stocks were prepared in

the same manner as human viruses except that there was no freeze/thaw step, and PRD1 was not chloroform extracted due to its 15% lipid volume by weight. Bacteriophage stocks were stored at -80 °C. Bacteriophage were assayed using the double agar layer (DAL) method with 100 µl sample inocula and modified Luria Bertani (LB) top and bottom agars. Modified LB consists of: bacto agar (0.75% (top) or 1.5% (bottom) wt/vol; BD), 10 g/L bacto tryptone (BD), 0.137 M NaCl, 1 g/L yeast extract (EMD Chemicals), 0.0055 M dextrose (EMD Chemicals), 0.002 M CaCl<sub>2</sub>. DAL plates were incubated at 37 °C for 18–24 h and PFUs were counted.

**Experimental Waters.** Viruses were inoculated into each of six different solutions: five environmental waters and a phosphate buffered saline (PBS) control. The environmental waters were collected in August 2009 as part of an associated field study.<sup>4</sup> Waters were stored at 4 °C in the dark and experiments were conducted within 8 months. The waters were as follows. MEX: mixture of seawater and partially treated wastewater collected near a coastal outfall in northern Mexico. BM: sewage-impacted seawater collected at a beach 0.5 km south of MEX. TJ: water collected from the Tijuana River estuary (Imperial Beach, California) at the end of ebb tide. ML: water collected from a section of the Malibu Lagoon coastal wetland (Malibu, CA) dominated by algae and submerged macrophytes. CT: water collected from a cattail dominated section of Malibu Lagoon. PBS consisted of 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 4 mM NaH<sub>2</sub>PO<sub>4</sub> and 10 mM NaCl. Previous research has shown that particles and colloids contribute to exogenous inactivation of MS2;<sup>9</sup> thus, environmental waters were not filtered or sterilized before use.

The absorption spectrum of each water sample was measured before and after experiments using a UV-visible spectrophotometer (scan from 250–800 nm; Lambda 35, Perkin Elmer). Turbidity and salinity were measured in MEX, BM, TJ and ML as part of the field study, using a Hach Hydrolab Quanta. pH was measured during simulated sunlight experiments using an Accumet pH electrode.

**Solar Simulator Experiments.** Experiment details are provided in the Supporting Information (SI). Experiments were conducted in duplicate 5-cm deep reactors containing 100 ml solution and exposed to a 1000 W solar simulator with a Xe bulb (Oriel). Viruses were spiked into experimental matrices and exposed to simulated sunlight for 10 h. Reactors were constantly mixed and maintained at 20 °C. One milliliter sub-samples were removed every 2 h, placed on ice and assayed within 6 h. Dark controls were maintained in the same way, but covered with aluminum foil.

Experiments were conducted using an atmospheric filter to mimic the solar spectrum or a UVB-blocking filter to remove the UVB portion of the spectrum. The average total irradiance from 280–700 nm was 194 W/m<sup>2</sup> (atmospheric filter) or 187 W/m<sup>2</sup> (UVB-blocking filter). Solar simulator spectra are presented in Figure S1.

**Singlet Oxygen.** Bulk-phase, steady-state singlet oxygen concentrations ( $[^1\text{O}_2]_{\text{ss,bulk}}$ ) were determined for each water through photolysis experiments using furfuryl alcohol (FFA)<sup>31</sup> as a probe compound. Photolysis experiments were conducted in a similar manner as virus inactivation experiments described above, with FFA added to each reactor at an initial concentration of 75  $\mu\text{M}$ . Detailed methodology is presented in the SI.

**Data Analysis.** First-order, observed inactivation rate constants for each virus in each matrix were calculated in two ways (detailed methodology is presented in the SI): (1)  $k_{\text{obs}}$  ( $\text{h}^{-1}$ ) was calculated as the negative slope of the linear regression trend line of  $\ln(C_t/C_0)$  *versus time*; and, (2)  $k_{\text{obs,photon}}$  ( $\text{m}^2 \text{photon}^{-1}$ ), was calculated as the negative slope of the linear regression trend line of  $\ln(C_t/C_0)$  *versus the average photon fluence in the water column* in the spectral range of 280–320 nm. Slopes, and their standard errors, were calculated using  $\ln(C_t/C_0)$  data pooled from duplicate experiments.

$k_{\text{obs,photon}}$  accounts for light screening by the water column, and provides a way to normalize inactivation rates across waters with different absorbance spectra. Photon fluence represents the number of photons available for absorption by the virus and photosensitizers, and was calculated over the range of 280–320 nm because this and previous research observed UVB light to be most important for virus inactivation.<sup>11</sup> A limitation of this, and similar, light screening calculations<sup>10,12,32</sup> is that we assume all wavelengths between 280 and 320 nm have the same ability to directly damage virions or produce ROS. While photons of different wavelengths have different abilities to damage viruses directly<sup>33</sup> and sensitize production of reactive species,<sup>34,35</sup> the complete information needed to develop weighting functions for each wavelength does not yet exist.

Unless noted otherwise, one-way ANOVA and Dunnett's post-test were performed to determine if  $k_{\text{obs}}$  in environmental waters were significantly different from  $k_{\text{obs}}$  in the sensitizer-free control. Statistical tests were performed in Prism (v5.04, GraphPad Software).

## Results



**Absorbance Spectra, pH, and Salinity.** As in other NOM-containing waters,<sup>22</sup> the absorbance of experimental waters used in this study was highest in the UVB region, and decreased with increasing wavelength (Figure S2). MEX absorbance measurements were 16 to 120 times greater than the other environmental waters (total absorbance over the range 280–700 nm), followed by BM > ML = CT > TJ > PBS. Total absorbance decreased by 8, 49, 39, 4 and 13% for MEX, BM, TJ, ML, and CT, respectively, during the 10 h experiments. pH ranged from 7.4–8.2 during experiments. Salinity ranged from 11.8–30.3 psu for the five environmental waters (Table S3).

**Singlet Oxygen.** Bulk-phase, steady-state singlet oxygen concentrations ( $[^1\text{O}_2]_{\text{ss,bulk}}$ ) were between  $(1.3 \pm 0.58) \times 10^{-14}$  and  $(8.3 \pm 0.55) \times 10^{-14}$  M, with TJ having the lowest and MEX the highest  $[^1\text{O}_2]_{\text{ss,bulk}}$  (Figure 2D; Table S3). While  $[^1\text{O}_2]_{\text{ss,bulk}}$  in PBS was calculated to be  $(1.2 \pm 1.1) \times 10^{-14}$  M, this value was not found to be statistically different than zero.

### **Simulated Sunlight Inactivation of Viruses and Bacteriophage.**

Inactivation curves with exposure to full-spectrum simulated sunlight are presented in Figure 1. Inactivation rates as functions of time ( $k_{\text{obs}}$ ) with exposure to full-spectrum and UVB-blocked sunlight are presented in Figures 2A and 2B, respectively, and inactivation rates as functions of photon fluence ( $k_{\text{obs,photon}}$ ) are presented in Figure 2C; numerical values, standard error and  $R^2$  of linear regressions are reported in Table S4. It should be noted that reported  $k_{\text{obs}}$  values are only applicable to the same exact conditions as in these experiments (e.g., same water composition, reactor depth, and sunlight spectrum and intensity).  $k_{\text{obs,photon}}$  values for all four viruses in MEX are likely overestimated. The tailing in the MEX absorbance spectrum indicates significant light scattering; this likely caused artificially high absorbance measurements and an overestimation of  $k_{\text{obs,photon}}$ .

Dark control inactivation rates ( $k_{\text{dark}}$ ) were always observed to be lower than the corresponding  $k_{\text{obs}}$  measured with exposure to simulated sunlight, except for HAdV2 exposed to UVB-blocked light in PBS, MS2 in PBS, and PV3 in CT (Table S4), though none of these  $k_{\text{dark}}$  values were found to be significantly different from zero.

**Poliovirus.** When exposed to full-spectrum simulated sunlight, PV3 was inactivated at the fastest rates in BM and the sensitizer-free control (PBS). PV3 inactivation in MEX, TJ, ML and CT occurred at significantly slower rates than in PBS ( $p < 0.001$  for MEX,  $p < 0.05$  for TJ, ML and CT); PV3  $k_{\text{obs}}$  was lowest in MEX. Slower inactivation rates in chromophore-containing waters suggest that light

attenuation, leading to fewer photons incident on virus nucleic acids and proteins, resulted in reduced PV3  $k_{obs}$ . This hypothesis is supported by analysis of inactivation rates after correcting for light screening (Figure 2C): there was no significant difference between PV3  $k_{obs,photon}$  in the sensitizer-free control and TJ, BM, ML, or CT.  $k_{obs,photon}$  was significantly larger in MEX ( $p<0.001$ ).

**PRD1.** When exposed to full-spectrum simulated sunlight, PRD1  $k_{obs}$  in environmental waters other than MEX were faster than  $k_{obs}$  in PBS, though not significantly different statistically. PRD1 inactivation in MEX, which had the highest absorbance, was significantly slower than in PBS ( $p<0.01$ ). After correcting for light screening, PRD1  $k_{obs,photon}$  in MEX, ML and CT were significantly faster than that in PBS ( $p<0.01$ ). The finding that PRD1 inactivation was enhanced in some waters containing light-absorbing natural constituents suggests that the exogenous mechanism contributed to inactivation.

**Adenovirus.** Inactivation of HAdV2 in ML, BM and TJ occurred at significantly faster rates than in PBS ( $p<0.05$ ,  $p<0.001$ , and  $p<0.001$ , respectively), with the greatest  $k_{obs}$  in TJ. While not statistically significant, HAdV2 inactivation in CT was also greater than in PBS. Sunlight inactivation of HAdV2 in PBS and MEX occurred at the slowest rates. After correcting for light screening, HAdV2  $k_{obs,photon}$  in MEX was greater than that in the other waters ( $p<0.001$ ), while  $k_{obs,photon}$  in the other environmental waters were not significantly different from that in PBS. As with PRD1, our results demonstrate the contribution of the exogenous mechanism in sunlight inactivation of HAdV2.

**MS2.** MS2 inactivation rates in TJ and MEX were similar to those in PBS, while inactivation in BM, ML and CT occurred at significantly faster rates ( $p<0.001$ ). After correcting for light screening, simulated sunlight inactivation in TJ and PBS occurred at similar rates while inactivation in MEX, ML and CT occurred at rates significantly faster than in PBS ( $p<0.01$ ). As with PRD1 and HAdV2, our results demonstrate the contribution of the exogenous mechanism in sunlight inactivation of MS2.

**UVB-blocking Experiments.** In experiments conducted with the UVB-blocking filter,  $k_{obs}$  ( $k_{obs,UVB-block}$ ) of all viruses in PBS were not significantly different from zero.  $k_{obs,UVB-block}$  of all viruses in environmental waters were found to be greater than those measured in PBS, though remained smaller than  $k_{obs}$  measured with exposure to full-spectrum sunlight (Table S4).  $k_{obs,UVB-block}$  values were significantly different from zero (ANCOVA;  $p<0.05$ ) for all viruses in all environmental waters, other than HAdV2 in MEX (Table S4).

**Relative Rates of Virus Inactivation.** Despite the dependence of  $k_{\text{obs}}$  on environmental conditions, PV3  $k_{\text{obs}}$  in every experimental water was significantly greater than  $k_{\text{obs}}$  of the other viruses in that water ( $p < 0.001$  for all, except  $p < 0.01$  for PV3 and PRD1 in MEX; ANOVA, Tukey's post-test; Figure 3). MS2 and HAdV2 were the most resistant to sunlight-mediated inactivation in all waters; MS2 and HAdV2  $k_{\text{obs}}$  were only significantly different from each other in TJ ( $p < 0.001$ ; ANOVA, Tukey's post-test), with MS2 inactivation slower than that of HAdV2.

## Discussion

**Mechanisms of Sunlight-mediated Inactivation.** There are two ways that virus inactivation mechanisms occur in sunlit surface waters: through (1) absorption of photons by the virus itself (direct and indirect endogenous inactivation) and (2) reaction with reactive species formed by photosensitizers in the water column (exogenous inactivation). Determining which mechanisms dominate inactivation for a specific virus is important for understanding how environmental conditions (e.g., sunlight irradiance, water quality, depth, mixing) affect  $k_{\text{obs}}$ .

The presence of photosensitizing molecules decreased the rate of sunlight-mediated inactivation of PV3, signaling that inactivation was dominated by endogenous mechanisms. On the other hand, the inactivation rates of HAdV2 and MS2 were enhanced in most environmental waters, indicating that the exogenous mechanism contributed; under the conditions studied, the net contribution of chromophores in the water to exogenous inactivation was greater than the decrease in endogenous inactivation due to light screening. The results for PRD1 were somewhere in between, with some evidence for exogenous inactivation.

All three inactivation mechanisms are wavelength dependent. The main components of non-enveloped viruses (proteins and nucleic acids) do not absorb light at wavelengths greater than ca. 320 nm,<sup>18,19</sup> which results in a reliance of endogenous mechanisms on UVB light. The exogenous mechanism, on the other hand, can be initiated by longer wavelengths due to the ability of NOM to absorb light into the visible region.<sup>22</sup> In experiments utilizing the UVB-blocking filter, we assume that all virus inactivation is attributed to the exogenous mechanism only. This assumption is validated by looking at  $k_{\text{obs,UVB-block}}$  in PBS, where exogenous inactivation is not possible due to the absence of exogenous photosensitizers;  $k_{\text{obs,UVB-block}}$  in PBS was not observed to be significantly different from zero for any of the viruses. This result agrees with previous studies by our lab that found PV3, HAdV2 and MS2 inactivation in sensitizer-free water to be due to UVB light alone.<sup>11,33</sup> One difference is that we

previously found that UVA light made a small contribution to endogenous PRD1 inactivation,<sup>11,33</sup> possibly due to PRD1's internal lipid membrane or the modified method used for its purification, which could potentially leave photosensitizers in the virus stock solution or attached to the virus.

$k_{\text{obs,UVB-block}}$  values were significantly different from zero for all viruses in all environmental waters, other than HAdV2 in MEX, indicating that all four viruses were susceptible to exogenous inactivation sensitized by UVA and visible light. Under full-spectrum sunlight, the contribution of the exogenous mechanism to inactivation of PV3 was not observable because endogenous mechanisms were so fast they masked the contribution of exogenous inactivation.

Differences in nucleic acid type and length *and* protein capsid composition and structure could account for the variability in observed endogenous and exogenous sunlight inactivation rates. DNA and RNA are well known targets of damage caused by UV light.<sup>18</sup> It has been postulated that the rate of genome damage depends on both the type of nucleic acid (i.e., single- or double-stranded DNA or RNA), and the length of the genome<sup>36</sup> or the number of sites susceptible to UV-photoreaction,<sup>37</sup> such as adjacent pyrimidines. Proteins are another potential target for endogenous sunlight damage: tyrosine, tryptophan and cysteine disulfide bonds absorb light in the UVB range,<sup>19</sup> and dissolved tyrosine and tryptophan have been found to undergo direct photolysis with exposure to simulated sunlight.<sup>20</sup> For exogenous inactivation,  $^1\text{O}_2$  was previously found to be more responsible for MS2 inactivation than  $\text{OH}\cdot$ ,  $\text{O}_2^-$  or  $\text{H}_2\text{O}_2$  in WSP water.<sup>9</sup> Recent work by Wigginton et al.<sup>38</sup> found  $^1\text{O}_2$  to cause significant MS2 genome decay, leading to inactivation of the virus. Proteins are also a potential target of exogenous sunlight damage due to their abundance in virus capsids, high reactivity with  $^1\text{O}_2$  and ability to bind exogenous photosensitizers.<sup>24</sup> HAdV, for example, may exhibit enhanced susceptibility to sunlight inactivation in waters containing photosensitizers due to the role of proteins as targets for photooxidative damage. Due to its double-stranded genome, HAdV is able to infect host cells despite DNA damage and then use host cell machinery to repair DNA and retain viability.<sup>39</sup> Damage to HAdV proteins, on the other hand, cannot be repaired and could therefore render the virus irreversibly inactivated.<sup>40</sup>

**The Role of NOM.** Variability in NOM structure can play a role in the variability in inactivation rates observed for each virus in different environmentally sourced waters. NOM has a complex structure that varies depending on its source, and affects its ability to absorb light, produce ROS, and interact with viruses.<sup>21,22,36,41</sup> While NOM in the water column can enhance sunlight inactivation of viruses susceptible to exogenously produced ROS, it can also inhibit all three sunlight inactivation mechanisms

by attenuating light or shielding viruses from radiation.<sup>41</sup> PV3 and PRD1  $k_{\text{obs}}$  in MEX, for example, were lower than  $k_{\text{obs}}$  in PBS (Figure 2A) due to the importance of endogenous mechanisms for these viruses.

For MS2 and HAdV2 inactivation in environmental waters, light attenuation was compensated for by increased damage through the exogenous mechanism, resulting in higher  $k_{\text{obs}}$  in some environmental waters compared to PBS. In full-spectrum simulated sunlight, Kohn and Nelson<sup>9</sup> had a similar finding for MS2 in WSP water, whereas Romero et al.<sup>12</sup> observed the opposite: the addition of 20 mg C/L of Suwannee River NOM (SRNOM) resulted in slower MS2 inactivation kinetics. The discrepancy between the Romero et al. finding and ours could be due to a higher fraction of UVB light emitted by their light source (6.24%, as compared to 0.81% in the present study), which would result in greater relative  $k_{\text{obs}}$  in clear water (i.e., endogenous  $k_{\text{obs}}$ ) as compared to  $k_{\text{obs}}$  in waters containing light-absorbing photosensitizers.

Given the importance of  $^1\text{O}_2$  in exogenous MS2 inactivation,<sup>9</sup> we investigated  $[^1\text{O}_2]_{\text{ss,bulk}}$  in the environmental waters. Natural waters have microheterogeneous  $[^1\text{O}_2]_{\text{ss}}$ , with higher concentrations closer to the source of production.<sup>28,42</sup> Thus, the effective  $[^1\text{O}_2]_{\text{ss}}$  that viruses are exposed to could be higher than  $[^1\text{O}_2]_{\text{ss,bulk}}$  if viruses are associated with photosensitizers. If virus-NOM association differs between environmental waters we could observe a non-linear relationship between  $k_{\text{obs,UVB-block}}$  corrected for light screening ( $k_{\text{obs,UVB-block,photon}}$ ;  $\lambda$  range 280-700 nm) and  $[^1\text{O}_2]_{\text{ss,bulk}}$ , depending on association affinities between the virus and NOM. This was not the case for MS2 inactivation in the present study, where we observed a linear relationship between  $[^1\text{O}_2]_{\text{ss,bulk}}$  and MS2  $k_{\text{obs,UVB-block,photon}}$  ( $R^2=0.91$ ; Figure 4). This finding agrees with Badireddy et al.,<sup>43</sup> who found MS2 inactivation by illuminated fullerenes to be fastest in solutions with the greatest  $[^1\text{O}_2]_{\text{ss,bulk}}$ , and Kohn and Nelson,<sup>9</sup> who found a linear relationship between  $[^1\text{O}_2]_{\text{ss,bulk}}$  and MS2  $k_{\text{obs}}$  in WSP water. The correlation between MS2  $k_{\text{obs,photon}}$  and  $[^1\text{O}_2]_{\text{ss,bulk}}$  observed in this study suggests that there are similar MS2-NOM association affinities between waters, resulting in  $[^1\text{O}_2]_{\text{ss,bulk}}$  being related to the  $[^1\text{O}_2]_{\text{ss}}$  experienced by MS2.

In contrast to MS2, HAdV2  $k_{\text{obs,UVB-block,photon}}$  did not correlate with  $[^1\text{O}_2]_{\text{ss,bulk}}$  ( $R^2=0.18$ ; Figure 4). Previous research by Dewilde et al.<sup>44</sup> and Pellieux et al.<sup>45</sup> investigated HAdV inactivation with exposure to  $^1\text{O}_2$  released by naphthalene endoperoxides (water-soluble compounds that thermally decompose to release  $^1\text{O}_2$ ) and found HAdV-endoperoxide interaction to be important. When exposed

to an endoperoxide that did not associate with the virus (1,4-naphthalenedipropionate), no HAdV inactivation was observed, even when exposed to  $^1\text{O}_2$  concentrations higher than those used in the present study.<sup>44</sup> However, when exposed to  $^1\text{O}_2$  released by an endoperoxide containing a nonionic functional group (N,N'-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropylamine), HAdV was inactivated efficiently,<sup>45</sup> likely due to HAdV-endoperoxide association. Differential binding affinity between HAdV2 and constituents in our experimental waters could account for the discrepancy between  $k_{\text{obs}}$  and  $[^1\text{O}_2]_{\text{ss,bulk}}$ .

Water quality conditions that increase virus-NOM binding affinity, such as high ionic strength and low pH,<sup>41</sup> have been observed to increase  $k_{\text{obs}}$ . Sinton et al.<sup>26</sup> and Kohn et al.,<sup>10</sup> for example, found greater sunlight inactivation rates of F+RNA phage and MS2, respectively, with increased ionic strength, presumably due to increased association between viruses and photosensitizers. Interestingly, we observed the fastest HAdV2  $k_{\text{obs}}$  values in waters with highest salinity (TJ and BM), but the same was not true for MS2. It should be noted that halides in seawater can also modify photosensitized processes by increasing both formation and quenching rates of ROS.<sup>46</sup>

**Viral Indicators of Sunlight Inactivation.** Consistent with previous findings in clear water,<sup>11</sup> we observed MS2 to be a conservative indicator for PV3 and HAdV2 inactivation in all experimental waters used in this study (Figure 3). Given that HAdV2 has been observed to be the most resistant virus to low pressure UVC light,<sup>47</sup> it is a significant finding that sunlight inactivation rates of MS2 did not exceed those of HAdV2. It has also been reported that MS2 was inactivated more slowly than porcine rotavirus in water with and without the addition of SRNOM.<sup>12</sup> For monitoring sunlight disinfection in sewage-influenced surface waters, WSP and constructed wetlands, it is not possible to monitor MS2 coliphage alone, but rather F+RNA coliphage in general. Love et al.<sup>11</sup> found that F+RNA coliphage isolates were inactivated faster than MS2 and HAdV2 in clear water, but slower than PV3, PRD1, F+DNA and somatic coliphage.

The viruses investigated in this study could behave differently in other environmental matrices or in deeper waters. For example, while PV3 was inactivated faster than PRD1, MS2 and HAdV2 in all matrices, MS2  $k_{\text{obs}}$  approached PV3  $k_{\text{obs}}$  in waters with greater UVB attenuation due to the importance of UVB in PV3 inactivation (Figure 3). In water deeper than our reactors (i.e., >5 cm), the inactivation rate of viruses like PV3, which rely heavily on UVB for inactivation, would decrease relative to viruses for which the exogenous mechanism and longer wavelengths contribute more. Additionally, in near-

surface waters, the main factor determining  $[^1\text{O}_2]_{\text{ss,bulk}}$  is the absorbance of the photosensitizers in that water, whereas in deeper waters where nearly all light is absorbed, quantum yield is the most important factor in determining  $[^1\text{O}_2]_{\text{ss,bulk}}$  averaged over depth.<sup>48</sup> The experimental system used in this study approximated near-surface water conditions and, as predicted by Haag and Hoigne,<sup>48</sup>  $[^1\text{O}_2]_{\text{ss,bulk}}$  was highest in the most absorbing water (MEX) and lowest in the least absorbing water (TJ). However, shallow water  $[^1\text{O}_2]_{\text{ss,bulk}}$  may not reflect average  $[^1\text{O}_2]_{\text{ss,bulk}}$  at depth, which could influence  $k_{\text{obs}}$  of viruses susceptible to exogenous damage.

Because sunlight mediated inactivation of viruses is affected by complex and variable environmental (sunlight spectrum and intensity), water quality (pH, DO, ionic strength, source and concentration of photosensitizers) and virus (genome type and number of sites susceptible to photo-transformation; protein capsid composition and structure) factors, accurately modeling virus inactivation rates is difficult, and the application and comparison of experimental values across studies is a challenge. To better model sunlight inactivation of viruses in the environment, and compare  $k_{\text{obs}}$  values between organisms in different waters, we need to better understand virus-NOM association, ROS production by NOM, and the photoaction spectra of viruses and NOM. In the meantime, studies should report sunlight spectra and water absorbance spectra, and care must be taken when comparing sunlight inactivation rates across studies. Also, in addition to reporting  $k_{\text{obs}}$  in units of inverse time, inactivation rates should be reported in terms of average light irradiance or photon fluence.

**Limitations.** Due to the time requirements of human virus inactivation experiments, experimental waters were used over the course of 8 months. There was little change in absorbance of these waters over that time, which is consistent with Zepp<sup>29</sup> who found no change to the absorbance spectrum of swamp water stored in the dark for two years. However, we cannot rule out that some changes occurred to the water matrices over time that affected the inactivation rates measured. Nonetheless, experiments with a particular virus were conducted in all waters at the same time and any changes would have minimal impact on relative inactivation rates.

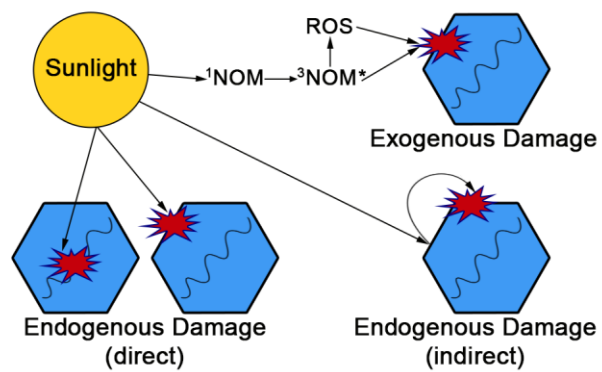
We were unable to measure whether viruses were aggregated in our experiments. MS2 was reported to exist as dispersed viruses in PBS at neutral pH.<sup>49,50</sup> While aggregation during the experiment could result in overestimation of  $k_{\text{obs}}$  attributed to sunlight, viruses already present in aggregates may result in underestimation of  $k_{\text{obs}}$  because the entire aggregate must be inactivated to prevent plaque formation.<sup>49,50</sup> In our experiments, viruses were added to reactors and stirred for 15 min in the dark

prior to collection of the  $t=0$  sample; controls indicated that minimal aggregation occurred after this time in the dark, but it is possible some aggregation occurred during the sunlight experiments.

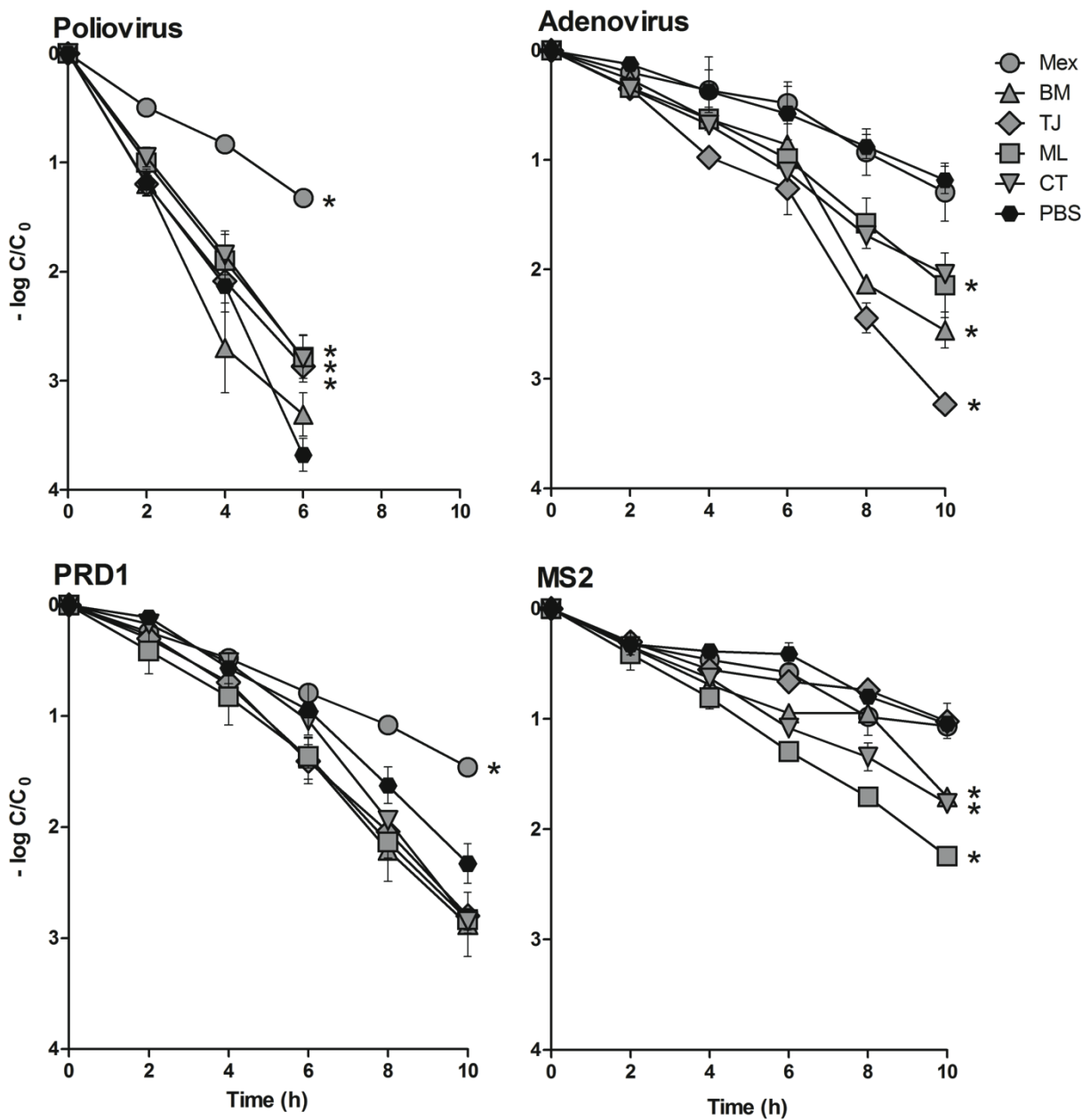
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**Supporting Information Available.** Detailed experimental and data analysis methodology; figures of simulated sunlight irradiance and water absorbance spectra; experimental water and virus characteristics; and, a summary of  $k_{\text{obs}}$  values are provided in the SI. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

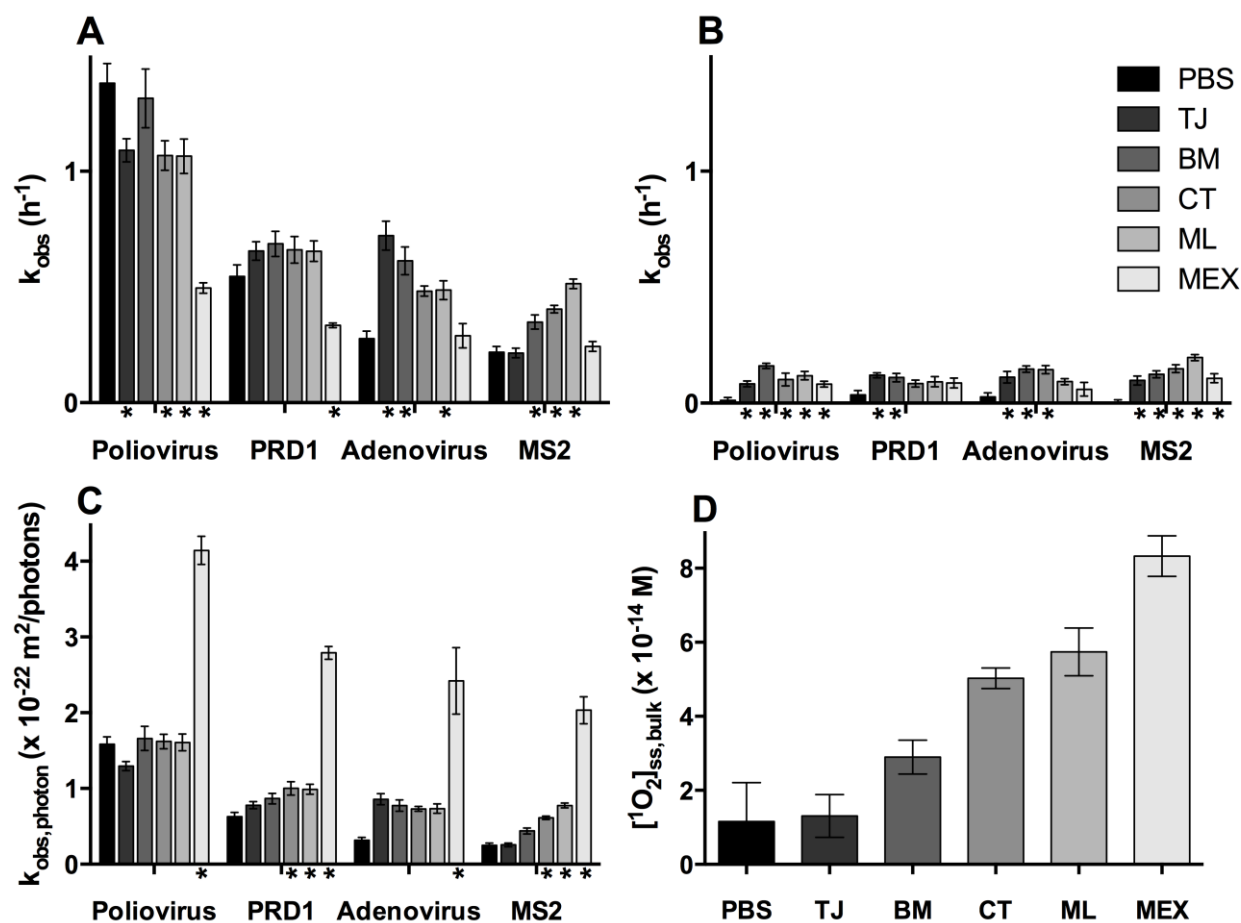




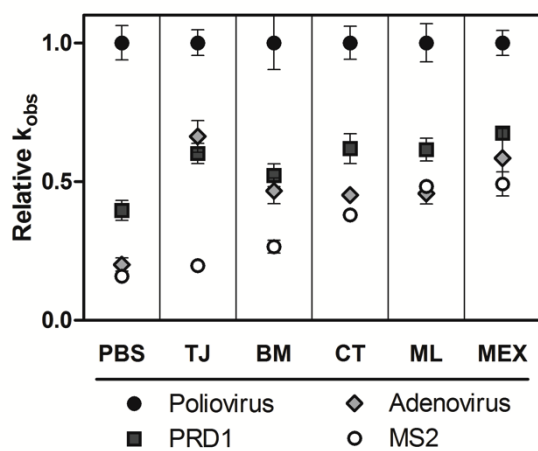
**Figure 1.** Solar simulator inactivation of PV3, HAdV2, MS2 and PRD1 inoculated into PBS or one of five environmental waters and exposed to full-spectrum simulated sunlight (experiment n=2, except n=1 for HAdV2 in CT). Error bars represent standard error. Asterisks indicate inactivation rates that differ significantly from PBS ( $p < 0.05$ ; Dunnett's post test).



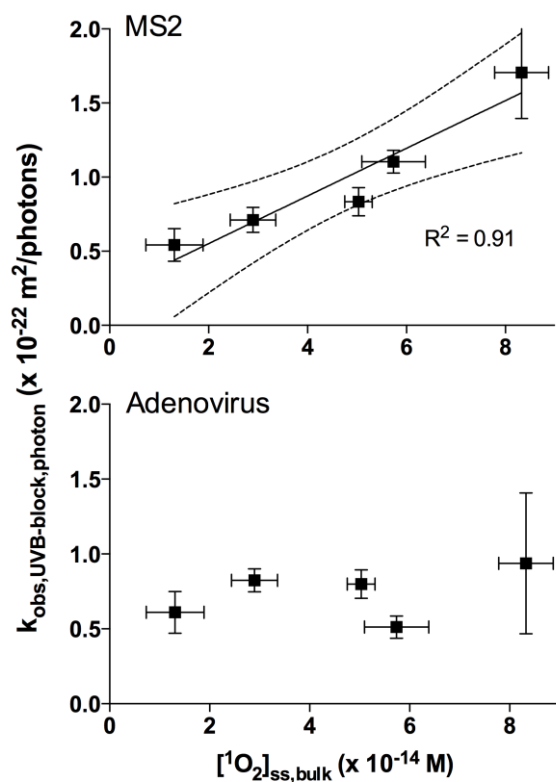
**Figure 2.** Panels A and B: First-order observed inactivation rates as functions of time ( $k_{\text{obs}}$ ;  $\text{h}^{-1}$ ) for viruses exposed to full-spectrum simulated sunlight (A) or simulated sunlight with the UVB region removed by an attenuation filter (B). Panel A was derived from data in Figure 1. Panel C: First-order observed inactivation rates as functions of photon fluence ( $k_{\text{obs,photon}}$ ;  $\text{m}^2 \text{photon}^{-1}$ ) with exposure to full-spectrum sunlight. Panel D: Bulk phase steady state singlet oxygen concentrations ( $[^1\text{O}_2]_{\text{ss,bulk}}$ ) with exposure to full-spectrum simulated sunlight. Waters are listed in order of increasing  $[^1\text{O}_2]_{\text{ss,bulk}}$ . Experiment n=2 for all, except n=1 for Panel D and HAdV2 in CT in Panel A. Error bars represent standard error. Asterisks indicate inactivation rates that differ significantly from PBS ( $p < 0.05$ ; Dunnett's post test).



**Figure 3.** Relative  $k_{\text{obs}}$  ( $\text{h}^{-1}$ ) of each virus in each matrix, as normalized by PV3  $k_{\text{obs}}$  in the same matrix. Waters are listed in order of increasing  $[^1\text{O}_2]_{\text{ss,bulk}}$ . Error bars are normalized standard error.



**Figure 4.** Solar simulator inactivation rates determined from UVB-blocking filter experiments and corrected for light screening ( $k_{\text{obs,UVB-block,photon}}$ ) versus bulk-phase, steady-state singlet oxygen concentrations ( $[^1\text{O}_2]_{\text{ss,bulk}}$ ) for MS2 and HAdV2. Inactivation in PBS was not included. Error bars represent standard error. Solid line in MS2 panel is the linear regression line; dotted lines are the 95% confidence intervals of the regression.



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